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Immunity to Enterotoxigenic *Escherichia coli*

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Enterotoxigenic *Escherichia coli* strains represent the most frequent etiological agent of travelers diarrhea. Challenge studies with several of these strains were undertaken in volunteers to evaluate the mechanisms of disease-induced immunity. Seventeen students and other community volunteers were given 10^6 or 10^8 organisms of *E. coli* B7A (O148:H28), which produces heat-labile and heat-stable enterotoxins. Ten individuals developed diarrheal illness closely resembling natural travelers diarrhea; of these ten, rises in titer of serum antitoxin and anti-O antibody occurred in eight (80%). Eight of the volunteers who developed diarrhea in the first test agreed to undergo rechallenge 9 weeks later with 10^8 B7A organisms. Only one of these eight "veterans" developed diarrhea versus seven of twelve controls given the same challenge ($P = 0.05$). Despite clinical protection, all "veterans" excreted B7A after rechallenge. Four controls who developed diarrhea during the homologous B7A rechallenge test were rechallenged 9 weeks later with 10^9 organisms of *E. coli* strain E2528-C1 (O25:H-), which produces only heat-labile enterotoxin and possesses a different O, H, and pili antigen composition than B7A. Three of four "veterans" and two of six controls developed comparable diarrhea. These studies demonstrate that prior disease due to enterotoxigenic *E. coli* confers homologous immunity against subsequent challenge, and the operative mechanism apparently is not bactericidal and is not mediated by serum anti-O antibodies. Heterologous protection was not conferred where the only common antigen was heat-labile enterotoxin, indicating that serum infection-derived antitoxin to heat-labile enterotoxin also is not protective.

Enterotoxigenic *Escherichia coli* (ETEC) strains are the most frequent etiological agents of short-incubation travelers diarrhea (19, 30, 45) and in some areas also appear important as a cause of endemic infantile diarrhea (8, 20). Prophylactic antibiotics have shown some efficacy in diminishing the risk of development of travelers diarrhea, including that due to ETEC (24, 37, 49). However, chemoprophylaxis has several drawbacks: (i) daily doses must be taken throughout the travel period; (ii) adverse drug reactions may occur; (iii) development of antibiotic-resistant bacterial strains may be encouraged; and (iv) normal intestinal flora are altered, perhaps leading to enhanced susceptibility to other enteropathogens such as *Shigella* and *Salmonella* (18, 22).

Immunoprophylaxis to control travelers diarrhea due to ETEC is appealing. A vaccine to prevent diarrhea due to ETEC will have to protect against numerous *E. coli* serotypes involving many O, H, and K antigens detected in pathogenic strains (11, 17, 30, 34, 38) that often possess several distinct virulence properties. Most ETEC strains, for example, elaborate heat-stable enterotoxin (ST) as well as heat-labile enterotoxin (LT) (30, 38). LT, which resembles cholera toxin pharmacologically and immunologically, is of high molecular weight, immunogenic, and neutralizable by LT or cholera antitoxin (13, 21, 31). ST, in contrast, is of low molecular weight, is not (or is minimally) immunogenic, and is not neutralized by LT or cholera antitoxin (1, 13, 21, 31). *E. coli* strains that elaborate only ST or LT are virulent for humans (28, 30, 39).

The studies of immunity to ETEC reported here were designed (i) to determine whether diarrheal infection due to a particular ETEC strain that produces both LT and ST would confer protection against subsequent challenge with the identical *E. coli* strain; (ii) To investigate whether individuals who experience diarrheal illness due to an *E. coli* strain that produces both LT and ST, and who develop an immunological response (documented by rises in circulating LT antitoxin and anti-O antibody), will be protected against subsequent challenge

with a heterologous *E. coli* strain of different antigenic composition that elaborates only LT; and (iii) to quantitate the circulating immune response to somatic O and LT antigens.

MATERIALS AND METHODS

Volunteers. Volunteers were college students and other community healthy adults (mean age, 25 years; range, 18 to 41). Challenge studies were carried out in the 22-bed Isolation Ward of the Center for Vaccine Development. The protocol was approved by the University of Maryland Hospital Human Volunteer Research Committee and the Clinical Review Sub-Panel of the National Institute of Allergy and Infectious Diseases. The studies were explained to volunteers in detail, and signed, witnessed consent was obtained. The informed nature of consent was documented prior to inoculation by requiring all volunteers to pass a written examination containing multiple-choice and true-false questions on all aspects of the study, including purpose, hazards, procedures, and pertinent bacteriology and immunology (26). The preinoculation health status of volunteers was ascertained from medical history, physical examination, chest radiograph, electrocardiogram, complete blood count, negative pregnancy test, urinalysis, blood chemistries (including serum glucose, urea nitrogen, and electrolytes), and tests for liver function, syphilis, and hepatitis B surface antigen.

Challenge strains. *E. coli* strain B7A (O148:H28), which elaborates LT and ST (LT+/ST+), was isolated from a United States soldier with diarrhea in Viet Nam (11). *E. coli* strain E2528-C1 (O25:NM) was responsible for an outbreak of diarrhea which involved several hundred persons on a cruise ship; this strain produces only LT (LT+/ST−) (5).

The two challenge strains were examined for the presence of colonization factor antigen I and II (CFA/I, CFA/II) pili and for type 1 somatic pili by hemagglutination tests and agglutination with specific antisera. After growth on solid CFA agar (14) for 24 h or after two 48-h stationary cultivations in Mueller-Hinton broth, the strains were tested for hemagglutinating properties against fresh human type A (14, 35), bovine (12), and guinea pig erythrocytes (3, 33, 35). Erythrocytes were washed twice with 0.85% saline and reconstituted as a 3% concentration in either 0.85% saline or 1.0% D-mannose. Multiple clones were picked from CFA agar plates with a sterile applicator stick and mixed on a clean glass slide with 0.025 ml of erythrocyte suspensions (12, 14). Broth-grown organisms were washed twice in 0.85% saline and reconstituted to a concentration of 10^{10} organisms/ml prior to testing on glass slides as above. Hemagglutination with human type A and guinea pig erythrocytes was carried out at 24°C (10, 14, 33, 35); tests with bovine cells were performed at 4°C by the method of Evans et al. (12). Hemagglutinations were graded from 0 to 4+, as described by Evans et al. (12, 14). Mannose-resistant hemagglutination of human type A and bovine cells was considered indicative of the presence of CFA/I and CFA/II, respectively, as described by Evans et al. (12, 14, 15). Mannose-sensitive hemagglutination of guinea pig cells provided evidence for the presence of type 1 somatic pili (3, 10, 33, 35, 42).

Neither challenge strain B7A nor E2528-C1 caused mannose-resistant hemagglutination of human type A or bovine erythrocytes after growth on solid CFA agar or in broth, indicating absence of CFA/I or CFA/II. Positive control strains H10407 (O78:H11) and B_2C (O6:H16), in contrast, caused 4+ mannose-resistant hemagglutination of human type A and bovine erythrocytes, respectively.

Both challenge strains, after 48-h stationary culture in Mueller-Hinton broth, manifested mannose-sensitive hemagglutination of human type A and guinea pig erythrocytes, indicative of the presence of "common" type 1 somatic pili.

Antibody to CFA/I of *E. coli* H10407, prepared by the method of Evans et al. (16), failed to agglutinate either challenge strain, corroborating the hemagglutination findings. Antibody to CFA/II was not available.

Antibody to type 1 somatic pili of *E. coli* H10407 (47) was kindly provided by C. C. Brinton, Jr., of the University of Pittsburgh. This antibody strongly agglutinated broth-grown H10407 and B7A, but only weakly (1+) agglutinated E2528-C1. When diluted to its end point (i.e., the highest dilution that still caused 4+ agglutination of *E. coli* H10407), the antibody caused 4+ agglutination of strain B7A but failed to agglutinate E2528-C1.

Type 1 somatic pili were visible on electron photomicrographs of strains B7A and E2528-C1 after growth in broth. However, the characteristic, thin, delicate CFA/I or CFA/II pili (12) were not visible on B7A or E2528-C1 after growth on CFA agar.

The challenge strains were noninvasive (negative in the guinea pig eye test) (44) and were sensitive to multiple antibiotics including ampicillin, neomycin, trimethoprim/sulfamethoxazole, kanamycin, gentamicin, and nalidixic acid.

Inocula and challenge. Inocula were prepared as previously described (11, 28). Briefly, 18-h Trypticase soy agar (BBL) cultures of the challenge strains were harvested with saline, and appropriate dilutions in saline were made. All volunteers drank 240 ml of water containing 2 g of $NaHCO_3$. One minute later, the inoculum (10^6, 10^8, or 10^9 organisms), which was suspended in 45 ml of phosphate-buffered saline (pH 7.2), was ingested. Fifteen minutes later, the volunteers (except three in the initial challenge group) drank another 240 ml of water containing 1 g of $NaHCO_3$. Volunteers were allowed nothing orally for 90 min before or after challenge. Inoculum size was quantitated by replicate pour-plate technique.

Clinical observations. Volunteers were examined daily beginning 2 to 3 days prior to inoculation. Oral temperatures were taken every 6 h and repeated within 5 min if they were 100°F (ca. 37.8°C) or above. All stools and and vomitus were collected in plastic cholera seats, examined, and graded, and the volume was measured by a nurse or physician. Stools were graded on a five-point scale (27) in which grades 1 and 2, representing fully formed and soft stools, respectively, are considered gradations of normal. Grade 3 describes thick liquid stool; grade 4 denotes opaque watery, and grade 5, rice water stools. Diarrhea was

defined as two or more loose (grade 3–5) stools in 24 h or at least one voluminous loose stool (>200 ml). Prior to discharge, all volunteers received a 5-day course of oral antibiotics (tetracycline or neomycin) to eradicate stool excretion of the pathogen.

Typing sera. Specific antisera for agglutination of the *E. coli* strains from stool cultures were prepared in rabbits as previously described (28).

Stool culture. Stool specimens or rectal swabs were inoculated on Levine's eosin-methylene blue (EMB) agar with and without appropriate antibiotic. Streptomycin or chloramphenicol was incorporated into EMB agar to facilitate recovery of organisms from volunteers who ingested B7A or E2528-C1, respectively; the strains were resistant to these respective antibiotics. Fifteen colonies (at least five from the agar plate without antibiotics) possessing a typical *E. coli* metallic sheen were picked and subcultured to slants of Trypticase soy agar in screw-top tubes. After 18 h of incubation at 37°C, the *E. coli* isolates were tested for agglutination with specific antiserum; the challenge *E. coli* strain served as a positive control and saline was used as a negative control. Slants were overlaid with mineral oil and stored at 4°C until tested for LT within 4 weeks.

Enterotoxin assays. *E. coli* cells from slants were inoculated into Syncase media (0.5 ml in vials) and incubated overnight at 37°C. Then 0.05 ml of the culture was added to monolayers of Y-1 adrenal cells in 96-well tissue culture plates (40). The cells were examined for rounding (evidence of LT enterotoxin) after 20 h of incubation at 37°C in 5% CO_2. Positive control strains included stock *E. coli* H10407.

Serology: passive hemagglutination for O antibody. Sera were collected prechallenge and 10 and 21 to 28 days postchallenge. Alkaline extract lipopolysaccharide O antigen from each challenge strain was prepared by the method of Young et al. (50); 0.1 ml of antigen was used to coat 1.0 ml of a 10% solution of glutaraldehyde-treated sheep erythrocytes (2). After heat inactivation (56°C, 30 min) and absorption with unsensitized sheep erythrocytes, antisomatic antibody was measured by a microtiter passive hemagglutination technique (25). Twofold dilutions of sera (0.025 ml) were added to microtiter wells followed by 0.025 ml of a 0.5% suspension of the sensitized sheep erythrocytes. Plates were incubated for 2 h at 37°C and 16 h at 4°C before they were read. High-titer lapine antisera from rabbits immunized with B7A or E2528-C1 served as positive controls.

Adrenal cell assay for LT antitoxin. Antitoxin to *E. coli* LT was assayed by a microtiter adrenal cell neutralization technique (7, 41). The reproducibility of this assay for *E. coli* LT antitoxin is comparable to our adrenal cell assay for measurement of cholera antitoxin (29). Filtered and lyophilized toxin from *E. coli* 408-3 (lot no. P-40) was kindly provided by J. Metzger of Fort Detrick, Md. Twofold dilutions of sera (0.025 ml), in triplicate, were incubated with equal volumes of *E. coli* enterotoxin (40 μg/ml) at 37°C for 1 h. The serum-toxin mixtures were then transferred to wells of microtiter plates containing monolayers of Y-1 adrenal cells. Plates were incubated in 5% CO_2 for 18 h at 37°C, and the wells were examined for rounding of cells.

Two reference standards were utilized in standardizing the assay: cell sensitivity and Swiss Serum and Vaccine Institute (SSVI; Berne, Switzerland) purified equine cholera antitoxin [lot no. EC3(A-2/67)-B; 4,470 antitoxin units per ml].

To measure cell sensitivity with each run of unknown sera tested, twofold serial dilutions of the toxin filtrate (40 μg/ml) were titrated concurrently with the test sera. The reciprocal end-point dilution (last well with 50% cell rounding) of the toxin was then divided into the initial toxin concentration. The sensitivity range was determined to be between 0.078 and 0.3 μg/ml, with most values equal to 0.156 μg/ml.

The purified equine cholera antitoxin was used to establish a standard neutralization end point as well as to assign antitoxin units to the test sera. SSVI control serum was diluted 1:62.5, after which further twofold serial dilutions were assayed with equal volumes of toxin filtrate on several different days. It was determined that a dilution of 1:250 was the standard end point of neutralization for 40 μg of *E. coli* 408-3 toxin filtrate per ml. Since the control serum has been given an arbitrary value of 1,000 antitoxin units/ml for *E. coli* LT antitoxin (41), the standard end-point concentration is equal to 4 antitoxin units/ml. This end point was constant for over 60 separate test runs during a 2-year period.

This control was included in each test run of unknowns. Antitoxin unit values for the unknowns were determined by comparing their end points to the control end point. Assuming that the end points of the unknowns and the control had an equal number of antitoxin units per milliliter, we multiplied that number of units by the reciprocal end point of each unknown to obtain antitoxin units per milliliter for each test specimen.

Antibody to type 1 somatic pili was not measured. Although intestinal fluid specimens were collected pre- and postchallenge, local intestinal antitoxin and anti-O antibody levels are not reported here because the techniques for their measurement are in the process of being refined. These results, when ready, will be presented in a subsequent publication.

Statistics. Differences in clinical attack rates were statistically evaluated by use of Fisher's Exact Test (4).

RESULTS

Dose-response studies with *E. coli* B7A. Two challenges were undertaken to identify the dose of ETEC organisms of strain B7A that would induce a clinical attack rate of approximately 60 to 70% (ID_{60} to ID_{70}) in healthy adult volunteers. As can be seen in Table 1, 3 of 6 volunteers who ingested 10^6 organisms (50%) and 7 of 11 who received 10^8 organisms (64%) developed diarrhea. The clinical and immunological responses of the 10 volunteers who developed illness are presented in Table 2 and demonstrate that the clinical spectrum of the volunteer disease model resembles that of naturally acquired travelers diarrhea.

Prior to challenge, no volunteers had *E. coli*

TABLE 1. *Clinical and immunological response of volunteers after oral challenge with E. coli B7A*

Inoculum size	No. of volunteers[a]	Diarrhea	Fever	Positive stool culture	Rise in circulating antibody ≧4×	
					Antitoxin	Antisomatic
10^6	6	3 (50%)	1 (17%)	6 (100%)	2 (33%)	2 (33%)
10^8	11	7 (64%)	2 (18%)	11 (100%)	8 (89%)[b]	8 (89%)[b]

[a] All volunteers received $NaHCO_3$ pretreatment.
[b] Only nine paired sera were available for testing.

TABLE 2. *Clinical and serological patterns of 10 volunteers who developed diarrheal illness after ingestion of 10^6 or 10^8 organisms of E. coli B7A*

Determination	Result
Incubation (h)	41.8 (14-124)[a]
Duration of illness (days)	3.6 (1-6.5)
Total no. of loose stools per volunteer	8.9 (1-25)
Peak no. of loose stools per volunteer per 24 h	3.7 (1-9)
Total stool volume per volunteer (ml)	1,124 (300-2,150)
Fever	30% (100^2-$101^{6°}$F)
Nausea	30%
Malaise	70%
Abdominal cramps	80%
Circulating antibody rises fourfold or greater	
Antitoxin	80%
Antisomatic	80%

[a] Mean with the range shown in parentheses.

strains in their stools that were agglutinated by B7A antisera. Within 24 to 48 h postingestion, all volunteers developed positive stool cultures, and *E. coli* B7A was the predominant coliform. Approximately one dozen *E. coli* clones that agglutinated with B7A antisera were randomly selected from each volunteer's stool cultures and tested for LT in the adrenal cell assay; 167 of 192 tested were positive (87%). Volunteers were not given antibiotics until the eighth day postchallenge to ensure an adequate antigenic stimulus.

Homologous B7A rechallenge. The 10 volunteers who developed diarrheal illness during the dose-response studies were asked to participate in a homologous rechallenge study, and 8 agreed to do so. Nine weeks after the initial B7A challenge, these 8 B7A "veterans" and 12 control volunteers ingested 10^8 *E. coli* B7A organisms. The results of this challenge are shown in Table 3. Only 1 of 8 "veterans" (13%) developed diarrheal illness versus 7 of 12 controls (58%). Despite the small numbers, this difference in attack rates was statistically significant ($P = 0.05$, Fisher's Exact Test; 4). The paucity of diarrheal illness in the 8 veterans upon rechallenge was in distinct contrast to their clinical response to initial challenge (Table 3).

Despite the protection against disease evident in the "veteran" group upon rechallenge, positive stool cultures were encountered in this group with the same frequency as in the control group. Quantitative cultures were not done. Onset of excretion occurred 1 day later in the rechallenge group, but the percentages of *E. coli* colonies picked that were agglutination positive (i.e., B7A) were the same in the stool cultures of the "veteran" and control groups.

The serum titers of antitoxic and anti-O antibody in the eight "veteran" volunteers in relation to the two challenges are displayed in Table 4. There were no correlations between these antibody levels and protection or susceptibility. The single "veteran" who developed illness upon rechallenge demonstrated (Table 4) an immunological response after initial challenge, with significant rises in antitoxic (eightfold) and anti-O (eightfold) circulating antibody titers. These titers diminished four- to eightfold from their peak to the time immediately prior to rechallenge.

Heterologous rechallenge study. Four volunteers who developed diarrhea while serving as controls in the B7A homologous rechallenge agreed to return 10 weeks later to participate in a heterologous rechallenge study. These four "veterans" and six control volunteers were fed 10^9 organisms of *E. coli* strain E2528-C1; this strain (O25:NM) produces LT but not ST and possesses somatic pili that are antigenically distinct from those of *E. coli* B7A. Dose-response studies with E2528-C1 and other LT+/ST− *E. coli* strains are reported elsewhere (M. M. Levine, D. R. Nalin, E. J. Bergquist, and R. B. Hornick, submitted for publication). Three of four "veterans" and two of six control volunteers developed diarrhea of comparable severity (Table 5).

Following their initial challenge with *E. coli* B7A, all four "veterans" developed fourfold or greater rises in circulating antitoxin (Table 6). However, by the time of rechallenge only two "veterans" still had serum antitoxin levels of 8 units/ml or above. After ingestion of *E. coli*

TABLE 3. *Response of 8 "veteran" volunteers upon initial challenge with E. coli B7A and upon subsequent rechallenge 9 weeks later in comparison with 12 control volunteers*

Group[a]	No. with diarrhea[b]	Incubation (h)[c]	Total diarrheal stool volume (ml)	Total loose stools	Nausea or vomiting	Malaise	Rise in antibody[d] (%)	
							Antitoxin	Antisomatic
Veterans (8 volunteers)								
Initial challenge	8 (100%)	44 (19–124)	7,035	30	3 (38%)	6 (75%)	75	75
Rechallenge	1 (13%)	52	557	9	0	0	50	13
Controls (12 volunteers)	7 (58%)	45 (17–68)	6,355	33	2 (17%)	7 (58%)	92	83

[a] All volunteers in both groups had positive stool cultures after challenge, whether or not they experienced diarrheal infection.

[b] Results of initial challenge versus rechallenge: $P = 0.002$. Results of rechallenge of veterans versus challenge of controls: $P = 0.05$.

[c] Mean with range given in parentheses.

[d] Fourfold or greater.

TABLE 4. *Serological response of volunteers following initial challenge and rechallenge with E. coli B7A*

Volunteers	Initial B7A challenge				B7A rechallenge				Diarrheal illness
	Antitoxin[a]		Antisomatic[b]		Antitoxin		Antisomatic		
	Pretiter	Peak titer	Pretiter	Peak titer	Pretiter	Peak titer	Pretiter	Peak titer	
"Veterans"[c]									
2004-1	16	128	64	128	128	128	128	64	−
2004-2	8	16	16	256	8	128	32	32	−
2004-3	16	1,024	128	512	64	256	128	64	−
2004-7	64	256	64	256	128	64	16	64	−
2004-9	<8	32	32	32	16	32	8	8	
2004-14	<8	8	128	512	<4	32	256	128	−
2004-16	8	64	16	128	8	64	32	64	+
2004-17	<8	1,024	16	512	256	256	8	16	−
Controls									
2005-5					<4	256	128	128	+
2005-6					<4	256	2	256	+
2005-11					16	256	8	32	+
2005-12					<4	128	32	256	+
2005-13					8	32	8	256	+
2005-14					16	128	16	128	−
2005-15					<4	16	2	16	−
2005-16					4	64	32	256	−
2005-17					32	128	16	256	+
2005-18					16	64	16	64	+
2005-20					16	32	16	128	−
2005-21					<4	32	16	16	−

[a] Antitoxin units per milliliter of serum.

[b] Reciprocal hemagglutination titer.

[c] All volunteers in the "veteran" group experienced diarrheal illness on initial challenge with *E. coli* B7A.

TABLE 5. *Heterologous challenge with E. coli E2528-C1 of volunteers who previously experienced diarrheal infection due to E. coli B7A in comparison with controls*

Group	No. of volunteers	No. with diarrhea	Incubation (h)	Total diarrheal stool volume (ml) per ill volunteer	Total no. of loose stools per ill volunteer
B7A "veterans"	4	3	19.7 (13–24)[a]	514 (301–622)	2.7 (1–5)
Control	6	2	16 (9.5–22.5)	482 (415–549)	4.5 (4–5)

[a] Mean with range given in parentheses.

TABLE 6. *Serological response of volunteers following ingestion of E. coli strains B7A and E2528-C1*

Volunteers	B7A challenge				E2528-C1 challenge				Diarrheal illness
	Antitoxin[a]		Antisomatic[b]		Antitoxin		Antisomatic[c]		
	Pre-titer	Peak titer	Pre-titer	Peak titer	Pre-titer	Peak titer	Pre-titer	Peak titer	
"Veterans"[d]									
2005-5	<4	256	128	128	64	64	128	256	+
2005-12	<4	128	32	256	<8	8	16	32	+
2005-13	8	32	8	256	<8	16	32	64	+
2005-17	32	128	16	256	8	16	64	64	−
Control									
2007-1					16	256	16	32	−
2007-2					<8	8	64	32	−
2007-3					32	256	32	64	−
2007-5					16	256	16	512	+
2007-11					<8	8	64	32	+
2007-13					16	128	64	256	−

[a] Antitoxin units per milliliter.
[b] Reciprocal hemagglutination titer against B7A antigen.
[c] Reciprocal hemagglutination titer against E2528-C1 antigen.
[d] All "veteran" volunteers experienced diarrheal illness on initial challenge with *E. coli* B7A.

E2528-C1, four of six controls developed rises in circulating antitoxin versus only one of four "veterans." All volunteers, both "veterans" and controls, had positive stool cultures.

DISCUSSION

E. coli B7A was selected as the main challenge organism because enterotoxigenic O148:H28 strains have been associated with travelers diarrhea in several geographically dispersed areas (11, 36). Despite the absence of CFA/I or CFA/II in this strain, within 24 to 48 h after challenge, *E. coli* B7A became the predominant coliform flora in stool cultures, the clinical illness mimicked natural Turista, and the volunteers had prominent serological responses to both toxin and somatic antigen (Tables 1 and 2).

The exact mechanism responsible for the immunity conferred by initial *E. coli* B7A infection is not clear. Possibilities include anti-LT, anti-O, or flagellar antibody and anti-adhesion factor antibody, either local or circulating. Since ETEC do not invade the mucosal surface, it is assumed that local, intestinal antibody of secretory immunoglobulin A (IgA) variety is probably highly important, but serum IgG and IgM antibody that leak onto the mucosal surface may also be involved in protection.

We consider it unlikely that anti-LT mediated the homologous immunity, since B7A also elaborates nonimmunogenic ST, which is not neutralized by LT antitoxin (13, 21, 31), and ST is a recognized virulence factor in human *E. coli* strains (28, 33). The precise role of anti-O, anti-adhesion factor, or antiflagellar antibodies in mediation of the observed immunity is not clear. It is possible that local antibody directed against adhesive factors possessed by the *E. coli* strain may have provided the operative mechanism of immunity by preventing attachment of *E. coli* to epithelial cells of the proximal small intestine, the anatomic site of enterotoxic diarrhea. Neither challenge strain produced CFA/I or CFA/II pili; however, both elaborated type 1 somatic pili. Although type 1 pili have been clearly shown to mediate attachment to epithelial cells in vitro (3, 23, 32, 43), the in vivo significance of this phenomenon in pathogenesis of ETEC diarrhea is not yet clarified. While LT+/ST+ ETEC strains often possess CFA/I or CFA/II, in our experience most LT+/ST− and LT−/ST+ strains (although virulent for humans) do not (M. M. Levine and V. Daya, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 18th, Atlanta, Ga., Abstr. no. 434, 1978). Most LT+/ST− and LT−/ST+ strains do elaborate type 1 somatic pili, but their exact role as organelles of attachment is still under investigation (3). For these reasons, it is not possible to favor the role of the immune response to type 1 somatic pili as the predominant mediator of immunity over the host response to O or H antigens. The observation that disease protection occurred in the face of positive stool cultures is explicable if protection was mediated by immunoglobulins within the secretory IgA class; IgA, although biologically active in other ways, is believed to be incapable of bactericidal activity in the gut environment (46, 48).

For the heterologous challenge study, *E. coli* E2528-C1 was selected because it had no detect-

able antigens with B7A except production of LT. This strain did not produce ST, was H−, and possessed a different O antigen. Although *E. coli* E2528-C1 has common type 1 somatic pili, they are antigenically distinct from B7A pili; antibody against *E. coli* H10407 type 1 somatic pili, which strongly agglutinates B7A, failed to agglutinate E2528-C1. Antibody produced in rabbits by inoculation with living B7A organisms also failed to agglutinate *E. coli* E2528-C1.

After recovery from B7A diarrhea, volunteers were not protected against diarrhea caused by E2528-C1. Since LT was the only antigen detected in both strains and all four "veterans" had developed significant rises in antitoxin following B7A diarrhea, the lack of protection implies that antitoxic antibodies were not an effective mode of immunity (6). However, this interpretation must be tempered by the observation that serum antitoxin levels in the four "veterans" involved in the heterologous rechallenge had declined from their peak to much lower levels by the time of challenge with E2528-C1 (Table 6). Local intestinal antitoxin may also have decreased to nonprotective levels.

Alternatively, LT of LT+/ST+ strains may differ from that of LT+/ST− strains and may not be neutralized by antitoxin against the former. In volunteers, the diarrheal syndrome associated with LT+/ST− *E. coli* is distinct from that due to LT+/ST+ strains (M. M. Levine, D. R. Nalin, E. J. Bergquist, and R. B. Hornick, submitted for publication). The incubation period of LT+/ST− strains is shorter, and diarrhea volume and duration are less. Against this hypothesis is the fact that volunteers challenged with E2528-C1 had rises in neutralizing antitoxin to LT derived from an LT+/ST+ strain (*E. coli* 408-3).

The observations reported here support the concept that ETEC infections may be preventable by immunological methods and establish a (relatively) low-inoculum volunteer model for testing candidate immunizing agents. The existence of homologous immunity in the absence of bactericidal mechanisms (individuals protected from disease excreted the challenge strain) suggests that the mechanism of immunity could be local intestinal. The specific mediators of the observed immunity are unclear and will require further investigation.

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LITERATURE CITED

1. **Alderette, J. F., and D. C. Robertson.** 1977. Purification and chemical characterization of the heat-stable enterotoxin produced by porcine strains of enterotoxigenic *Escherichia coli*. Infect. Immun. **19**:1021–1030.
2. **Bing, D. H., J. G. M. Weyand, and A. B. Stavitsky.** 1967. Hemagglutination with aldehyde-fixed erythrocytes for assays of antigens and antibodies. Proc. Soc. Exp. Biol. Med. **124**:1166–1170.
3. **Brinton, C. C., Jr.** 1977. The piliation phase syndrome and the uses of purified pili in disease control, p. 33–70. *In* Proceedings of the 13th Joint U.S.-Japan Conference on Cholera, Atlanta, Ga., September 1977. Department of Health, Education and Welfare Publication no. 78-1590. National Institutes of Health, Bethesda, Md.
4. **Brownlee, K. A.** 1962. Statistical method and theory in science and engineering. John Wiley & Sons, Inc., New York.
5. **Center for Disease Control.** 1976. Diarrheal illness on a cruise ship caused by enterotoxigenic *Escherichia coli*. Morbid. Mortal. Wkly. Rep. **25**:229.
6. **Curlin, G., R. Levine, A. Aziz, A. S. M. Rahman, and W. F. Verwey.** 1976. Field trial of cholera toxoid, p. 314–329. *In* Proceedings of the 11th Joint Conference of the U.S.-Japan Cooperative Medical Science Program Symposium on Cholera, New Orleans, 1975. National Institutes of Health, Bethesda, Md.
7. **Donta, S. T., D. A. Sack, R. B. Wallace, H. L. DuPont, and R. B. Sack.** 1974. Tissue-culture assay of antibodies to heat-labile *Escherichia coli* enterotoxins. N. Engl. J. Med. **291**:117–121.
8. **Donta, S. T., R. B. Wallace, S. C. Whipp, and J. Olarte.** 1977. Enterotoxigenic *Escherichia coli* and diarrheal disease in Mexican children. J. Infect. Dis. **135**:482–485.
9. **Duguid, J. P.** 1964. Functional anatomy of *Escherichia coli* with special reference to enteropathogenic *E. coli*. Rev. Latinoam. Microbiol. **7**(Suppl. 13–15):1–6.
10. **Duguid, J. P., I. W. Smith, G. Dempster, and P. N. Edmunds.** 1955. Non-flagellar filamentous appendages ("Fimbriae") and haemagglutinating activity in *Bacterium coli*. J. Pathol. Bacteriol. **70**:335–348.
11. **DuPont, H. L., S. B. Formal, R. B. Hornick, M. J. Synder, J. P. Libonati, D. G. Sheahan, E. H. LaBrec, and J. P. Kalas.** 1971. Pathogenesis of *Escherichia coli* diarrhea. N. Engl. J. Med. **285**:1–9.
12. **Evans, D. G., and D. J. Evans, Jr.** 1978. New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups O6 and O8. Infect. Immun. **21**:638–647.
13. **Evans, D. G., D. J. Evans, Jr., and N. F. Pierce.** 1973. Differences in the response of rabbit small intestine to heat-labile and heat-stable enterotoxins of *Escherichia coli*. Infect. Immun. **7**:873–880.
14. **Evans, D. G., D. J. Evans, Jr., and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18**:330–337.
15. **Evans, D. G., D. J. Evans, Jr., W. S. Tjoa, and H. L. DuPont.** 1978. Detection and characterization of the colonization factor of enterotoxigenic *Escherichia coli* isolated from adults with diarrhea. Infect. Immun. **19**: 727–736.
16. **Evans, D. G., R. P. Silver, D. J. Evans, Jr., D. G. Chase, and S. L. Gorbach.** 1975. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. **12**:656–667.
17. **Evans, D. J., Jr., D. G. Evans, H. L. DuPont, F.**

Orskov, and I. Orskov. 1977. Patterns of loss of enterotoxigenicity by *Escherichia coli* isolated from adults with diarrhea: suggestive evidence for an interrelationship with serotype. Infect. Immun. **17**:105–111.

18. **Freter, R.** 1956. Experimental enteric shigella and vibrio infections in mice and guinea pigs. J. Exp. Med. **104**: 411–418.
19. **Gorbach, S. L., B. H. Kean, D. G. Evans, D. J. Evans, Jr., and D. Bessudo.** 1975. Travelers' diarrhea and toxigenic *Escherichia coli*. N. Engl. J. Med. **292**: 933–936.
20. **Guerrant, R. L., R. A. Moore, P. M. Kirschenfeld, and M. S. Sande.** 1975. Role of toxigenic and invasive bacteria in acute diarrhea of childhood. N. Engl. J. Med. **293**:567–573.
21. **Gyles, C. L.** 1971. Heat-labile and heat-stable forms of the enterotoxin from *E. coli* strains enteropathogenic for pigs. Ann. N.Y. Acad. Sci. **196**:314–322.
22. **Hornick, R. B., S. E. Greisman, T. E. Woodward, H. L. DuPont, A. T. Dawkins, and M. J. Synder.** 1970. Typhoid fever: pathogenesis and immunologic control. N. Engl. J. Med. **283**:686–691, 739–741.
23. **Isaacson, R. F., P. C. Fusca, C. C. Brinton, and H. W. Moon.** 1978. In vitro adhesion of *Escherichia coli* to porcine small intestinal epithelial cells: pili as adhesive factors. Infect. Immun. **21**:392–397.
24. **Kean, B. H.** 1963. The diarrhea of travelers to Mexico: summary of five-year study. Ann. Intern. Med. **59**: 605–614.
25. **Lee, M. R., N. C. Ikari, N. C. Branche, Jr., and V. M. Young.** 1966. Microtiter bacterial hemagglutination technique for detection of Shigella antibodies. J. Bacteriol. **91**:463.
26. **Levine, M. M.** 1976. Legal and ethical problems in epidemiologic research—informed consent. Am. J. Epidemiol. **104**:350.
27. **Levine, M. M., E. J. Bergquist, D. R. Nalin, D. H. Waterman, R. B. Hornick, C. R. Young, and S. Sotman.** 1978. *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are noninvasive. Lancet **i**:1119–1122.
28. **Levine, M. M., E. S. Caplan, D. Waterman, R. A. Cash, R. B. Hornick, and M. J. Synder.** 1977. Diarrhea caused by *Escherichia coli* that produce only heat-stable enterotoxin. Infect. Immun. **17**:78–82.
29. **Levine, M. M., T. P. Hughes, C. R. Young, S. O'Donnell, J. P. Craig, H. P. Holley, and E. J. Bergquist.** 1978. Antigenicity of purified glutaraldehyde-treated cholera toxoid administered orally. Infect. Immun. **21**:158–162.
30. **Merson, M. H., G. K. Morris, D. A. Sack, J. G. Wells, J. C. Feeley, R. B. Sack, W. B. Creech, A. Z. Kapikian, and E. J. Gangarosa.** 1976. Travelers' diarrhea in Mexico: a prospective study of physicians and family members attending a congress. N. Engl. J. Med. **294**: 1299–1305.
31. **Nalin, D. R., A. K. Bhattacharjee, and S. H. Richardson.** 1974. Cholera-like toxin effect of culture filtrates of *Escherichia coli*. J. Infect. Dis. **130**:595–601.
32. **Ofek, I., D. Mirelman, and N. Sharon.** 1977. Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. Nature (London) **265**:623–625.
33. **Old, D. C.** 1972. Inhibition of the interaction between fimbrial haemagglutinations and erythrocytes by D-mannose and other carbohydrates. J. Gen. Microbiol. **71**:149–157.
34. **Orskov, F., I. Orskov, D. J. Evans, Jr., R. B. Sack, D. A. Sack, and T. Wadstrom.** 1976. Special *Escherichia coli* serotypes among enterotoxigenic strains from diarrhea in adults and children. Med. Microbiol. Immunol. **162**:73–80.
35. **Orskov, I., and F. Orskov.** 1977. Special O:K:H serotypes among enterotoxigenic *E. coli* strains from diarrhea in adults and children. Occurrence of the CF (colonization factor) antigen and of hemagglutinating abilities. Med. Microbiol. Immunol. **163**:99–110.
36. **Rowe, B.** 1974. *Escherichia coli* 0148 and diarrhoea in adults. Br. Med. J. **3**:741.
37. **Sack, D. A., D. C. Kaminsky, R. B. Sack, J. N. Itotia, R. R. Arthur, A. Z. Kapikian, F. Orskov, and I. Orskov.** 1978. Prophylactic doxycycline for travelers' diarrhea. Results of a prospective double blind study of Peace Corps volunteers in Kenya. N. Engl. J. Med. **298**: 758–763.
38. **Sack, D. A., J. C. McLaughlin, R. B. Sack, F. Orskov, and I. Orskov.** 1977. Enterotoxigenic *Escherichia coli* isolated from patients at a hospital in Dacca. J. Infect. Dis. **135**:275–280.
39. **Sack, D. A., M. H. Merson, J. G. Wells, R. B. Sack, and G. K. Morris.** 1975. Diarrhea associated with heat-stable enterotoxin-producing strains of *Escherichia coli*. Lancet **ii**:239–241.
40. **Sack, D. A., and R. B. Sack.** 1975. Test for enterotoxigenic *Escherichia coli* using Y-1 adrenal cells in miniculture. Infect. Immun. **11**:334–336.
41. **Sack, R. B., N. Hirschhorn, W. Woodward, D. A. Sack, and R. A. Cash.** 1975. Antibodies to heat-labile *Escherichia coli* enterotoxin in Apaches in Whiteriver, Arizona. Infect. Immun. **12**:1475–1477.
42. **Salit, I. E., and E. C. Gotschlich.** 1977. Hemagglutination by purified type 1 *Escherichia coli* pili. J. Exp. Med. **146**:1169–1181.
43. **Salit, I. E., and E. C. Gotschlich.** 1977. Type 1 *Escherichia coli* pili: characterization of binding to monkey kidney cells. J. Exp. Med. **146**:1182–1194.
44. **Sereny, B.** 1957. Experimental kerato-conjunctivitis shigellosa. Acta Microbiol. Hung. **4**:368–376.
45. **Shore, E. G., A. G. Dean, K. J. Holik, and B. R. Davis.** 1974. Enterotoxin-producing *Escherichia coli* and diarrheal disease in adult travelers: a prospective study. J. Infect. Dis. **129**:577–582.
46. **South, M. A.** 1971. Enteropathogenic *Escherichia coli* disease: new developments and perspectives. J. Pediat. **79**:1–11.
47. **Swaney, L. M., Y.-P. Liu, K. Ippen-Ihler, and C. C. Brinton, Jr.** 1977. Genetic complementation analysis of *Escherichia coli* type 1 somatic pilus mutants. J. Bacteriol. **130**:506–511.
48. **Tomasi, T. B., Jr.** 1972. Secretory immunoglobulins. N. Engl. J. Med. **287**:500–506.
49. **Turner, A. C.** 1967. Travellers' diarrhoea: a survey of symptoms, occurrence and possible prophylaxis. Br. Med. J. **4**:653–654.
50. **Young, V. M., M. R. Sochand, and H. C. Gillem.** 1960. Infectious agents in infant diarrhea. I. A hemagglutination-inhibition procedure for detection of bacterial fractions in infant sera. Proc. Soc. Exp. Biol. Med. **105**: 635–638.